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# REPETITIVE MOLECULAR EXCLUSION CHROMATOGRAPHY OF $PGB_X$ ON SEPHADEX LH-20

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$PGB_X$, a mixture of oligomers of 15-keto $PGB_1$, was subjected to repetitive MEC on Sephadex LH-20 in an attempt to separate and purify the active principle. The resulting fractions were analyzed and found still to be hetereogeneous. Although the MEC technique did not resolve the $PGB_X$ complex, low molecular weight fractions were obtained that may be amenable to mass spectral analysis.

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# L I S T  O F  A B B R E V I A T I O N S

MEC - Molecular Exclusion Chromatography

$\bar{M}n$ - Number Average Molecular Weight

RLM - Rat Liver Mitochondria

Type II $PGB_x$ - Fraction 2 of 1st Sephadex LH-20 MEC

VPO - Vapor pressure osmometry

## INTRODUCTION

$PGB_X$, a polymeric derivative of 15-keto $PGB_1$, was first synthesized by Polis et al (1) and shown to conserve oxidative phosphorylation during degradation of isolated rat liver mitochondria (2). More recently Ohnishi and Devlin (3) showed that $PGB_X$ behaved as a water soluble $Ca^{++}$-ionophore in skeletal muscle sarcoplasmic recticulum. Although these properties of $PGB_X$ are significant, the major interest in this compound is its possible role as a therapeutic agent. This is based on the animal studies of (a.) Polis and Angelakos (4) in which $PGB_X$ treated monkeys survived cardiogenic shock, (b.) Polis and Kolata (5) in which $PGB_X$ treated rabbits survived experimentally induced global ischemia, and (c.) Moss et al (6) in which $PGB_X$ treated dogs survived experimentally induced "fatal" hypoxia.

$PGB_X$ prepared according to Polis et al is a mixture of oligomers of varying molecular weight and unknown chemical composition. Before human testing may be undertaken, the active principle in the $PGB_X$ complex must be isolated in pure form and it chemical structure determined. Studies are currently underway in this and other laboratories to attain the above goal. This report describes the use of repetitive MEC of $PGB_X$ on Sephadex LH-20 in order to obtain the $PGB_X$ complex as a narrower molecular weight range of oligomers and thus a purer preparation.

## MATERIALS AND METHODS

The $PGB_X$ used in this study was prepared according to Polis et al (2) and was the crude extract before purification by MEC. When Sephadex LH-20 MEC was carried out, the procedure used was as described before (2). Molecular weights ($\bar{M}n$) were determined by VPO on the free acids dissolved in methanol, using a Wescan Molecular Weight Apparatus (Wescan, Santa Clara, CA) at 60°. Purified fractions were assayed for the in vitro $PGB_X$ effect on the stabilization of oxidative phosphorylation during degradation of RLM by the method of Polis et al (7) as modified by Shmukler et al (8). This modification includes the definition and measurement of the $PGB_X$ $K_a$ and $K_i$ effects. UV absorption spectra of $PGB_X$ fractions were measured in a Cary Recording Spectrophotometer at a concentration of 30µg/ml dissolved in methanol.

## RESULTS

In the original method for the preparation of $PGB_X$, Polis et al (2) used Sephadex LH-20 MEC in an attempt to purify the active principles of the $PGB_X$ complex. Although the exclusion limit for Sephadex LH-20 is between 100-500 daltons, no clean chromatographic separations were obtained. Instead the $PGB_X$ complex eluted as one diffuse chromatographic peak. In order to effect some degree of separation, Polis et al arbitrarily collected effluent fractions which were then monitored for their in vitro RLM oxidative phosphorylation effect.

The most active $PGB_X$ preparation, fraction 2[1], gave a $\bar{M}n$ of 2200-2300 by VPO. Since this fraction was the $PGB_X$ fraction sent to Office of Naval Research contractors for testing, it was of interest to try to purify this fraction even further. As a possible method to accomplish this, repetitive Sephadex LH-20 MEC was tried in order to purify the active principle and/or to obtain a $PGB_X$ preparation with a narrower molecular weight range of components than the Type II $PGB_X$.

In this study 6620 mg of crude extract of $PGB_X$, i.e., the $NAHCO_3$ extract (2) were converted to the free acid, dissolved in methanol and chromatographed on Sephadex LH-20 as described by Polis et al (2). The resulting seven fractions were flash evaporated and analyzed for weight recovery, $\bar{M}n$ and in vitro $PGB_X$ effects. These results are listed in table I. As seen in this table, fractions 1-2[2] and fraction 1-3 had approximately the same in vitro $PGB_X$ activity, therefore these 2 fractions were combined in order to have sufficient material for further MEC and subsequent analyses. This combination contained 1932 mg of $PGB_X$ of which 1385 mg were used in the 2nd MEC. Seven fractions were separated and analyzed as above. These results are listed in table II. As seen in this table fractions 2, 3, 4, and 5 all had approximately the same level of $PGB_X$ activity, although the Mn values were markedly different. For the 3rd MEC fractions, 2-2 and 2-3 were combined (679 mg) and the analyses of the resulting seven fractions are listed in table III. The separation methodology used in this study is summarized by schematic representation shown in figure 1.

The overall weight recovery of $PGB_X$ in the 1st MEC was 88.5 percent. This suggests that 11.5 percent of the crude extract was either irreversibly absorbed to the column, or adsorbed strongly to the column to elute outside the $PGB_X$ range. The overall weight recovery of the 2nd and 3rd MEC was approximately quantitative, i.e., 95.7 and 101.9 percent respectively. This suggests that material not recovered in the 1st MEC is not $PGB_X$.

Chromatography of the crude $PGB_X$ extract, 1st MEC, yields fractions varying in $\bar{M}n$ from 3049 to 372 as the retention time of the eluted material increased. Rechromatography of fractions 1-2 and 1-3 (2nd MEC) yielded fractions with $\bar{M}n$ varying from 2873 to 407. Even after 3rd MEC of fractions 2-2 and 2-3, the $\bar{M}n$ of the fractions varied from 2062-1466. Although the $\bar{M}n$ for fraction 3-7 could not be measured because of insufficient quantity, it would be reasonable to assume that the $\bar{M}n$ of fraction 3-7 would be similar to that of fraction 2-7.

The dry weights of all fractions separated in the repetitive MEC listed in tables I, II, and III were used to calculate the percent distribution of $PGB_X$ in each fraction in each separate MEC. These results are listed in table IV. The percent distribution of $PGB_X$ in the 1st MEC is approximately evenly distributed between fractions 1 through 6. On rechromatography of fractions 2-2 and 2-3, 59 percent of the $PGB_X$ was found in fractions 3-2 and 3-3.

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[1]Fraction 2 of the 1st Sephadex LH-20 MEC is referred to as Type II $PGB_X$.

[2]Fractions separated by repetitive MEC are described by 2 numbers: 1st number refers to MEC No.; 2nd number refers to MEC fraction. Thus 1-2 describes fraction 2 from 1st MEC which is equivalent to Type II $PGB_X$.

The in vitro $PGB_x$ assay data listed in tables I, II and III show that the $K_a$ was highest in fractions 2, 3 and 4 of the 1st MEC. Fraction 1-1 was about 80 percent pure while fractions 1-5, 1-6, and 1-7 were relatively impure. Rechromatography of fractions 1-2 and 1-3 (2nd MEC) showed that the $K_a$ was equally spread throughout fractions 2-2 to 2-6 with fractions 2-1 and 2-7 exhibition low values. When fractions 2-2 and 203 were rechromatographed (3rd MEC), only fractions 3-7 showed a low level of $K_a$. The results of the $K_i$ distribution in the various fractions were similar to those found for the $K_a$ distribution.

The UV absorption spectra between 200 nm to 400 nm were measured for all fractions separated in this study. In general, all fractions showed absorption maxima at 243 nm and absorption shoulders at 300- 320 nm. The major difference between the UV abosrption spectra of the separated fractions was the increase in the 300- 320 nm absorption shoulder with increasing retention time of the fractions. The results are listed in table V in terms of the ratio of the absorbance at 243 nm to absorbance at 310 nm. A comparison of the retention time and the $A_{243}/A_{310}$ shows a progressive decrease (or increase in $A_{310}$) with increasing retention time. A comparison of the $A_{243}/A_{310}$ of fractions with the same retention time but successive MEC, i.e., fractions 1-2 and fraction 2-2, shows a marked increase in the ratio (or a marked decrease in $A_{310}$). However, the data for the 2nd and 3rd MEC showed no change in the $A_{243}/A_{310}$ for fractions with the same retention time.

## D I S C U S S I O N

The results of repetitive MEC of $PGB_x$ on Sephadex LH-20 shows that this method does not yield homogeneous $PGB_x$ preparations as might have been expected. Instead, the fractions that were separated still appear to be hetereogeneous even after 3 MEC analyses. These results suggest that possibly even after additional MEC of fractions 3-2 and 3-3, homogeneous preparations of $PGB_x$ would not be obtained. From these results it is obvious that Type II $PGB_x$, the preparation currently supplied ONR contractors for in vitro and in vivo animal studies, is a highly complex mixture of oligomers varying in $\bar{M}n$ from over 2800 to below 400 daltons. It is interesting to note also that the $PGB_x$ fractions separated did not show a significant increase in the specific activity of the $PGB_x$, i.e., $K_a$.

One benefit realized with the repetitive MEC is that the $PGB_x$ fractions separated in MEC #3 must have a narrower range of molecular weight components than found in Type II $PGB_x$. An additional advantage is the recovery of high $K_a$ activity in relatively low molecular weight fraction, e.g. fraction 3-6 that had the following analytical values; $\bar{M}n$, 1466; $K_a$ 0.93, $K_i$ 0.92. It is conceivable that such a low molecular weight preparation may be amenable to high resolution and/or field desorption mass spectral analysis.

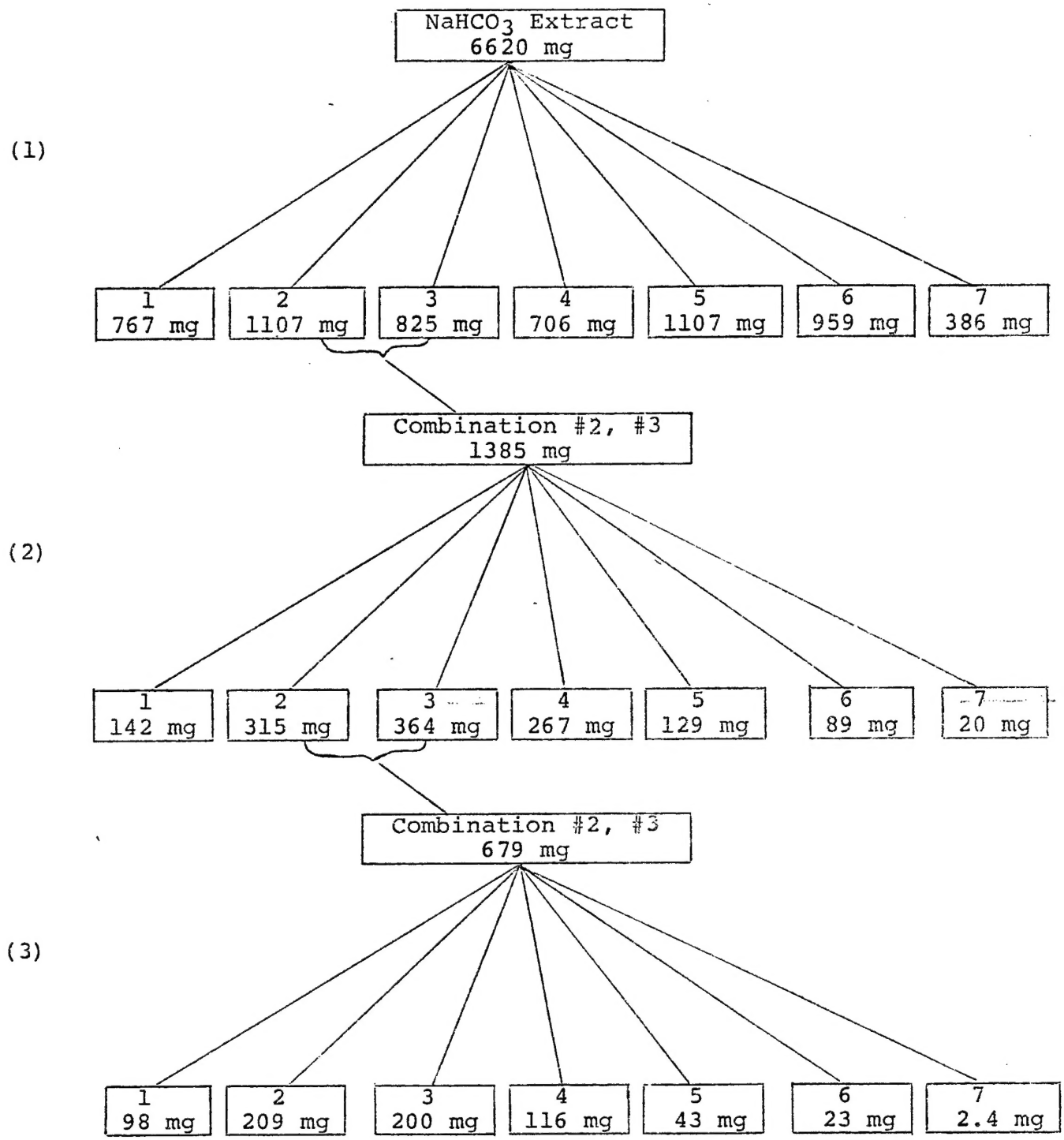


Figure 1 - Schematic Representation of Methodology for Repetitive MEC of $PGB_X$ on Sephadex LH-20

Table I

Distribution of $PGB_x$ in Sephadex LH-20

Fractions: 1st MEC

| Fraction | $\bar{M}n$ | Wt (mg) | $K_a$ | $K_i$ |
|---|---|---|---|---|
| 1 | 3049 | 767 | .82 | 1.18 |
| 2 | 2554 | 1107 | .97 | 1.33 |
| 3 | 2137 | 825 | 1.02 | 1.48 |
| 4 | 1706 | 706 | .98 | 1.36 |
| 5 | 1257 | 1107 | .47 | .81 |
| 6 | 915 | 959 | .33 | .40 |
| 7 | 372 | 386 | .30 | .12 |
| Total Recovery | 5858 mg | | | |
| % | 88.5 | | | |

Table II

Distribution of $PGB_X$ in Sephadex LH-20

Fractions: 2nd MEC

| Fraction | $\bar{M}n$ | Wt (mg) | $K_a$ | $K_i$ |
|---|---|---|---|---|
| 1 | 2873 | 142 | .31 | .67 |
| 2 | 2683 | 315 | .92 | 1.04 |
| 3 | 2351 | 364 | 1.06 | 1.08 |
| 4 | 2190 | 267 | 1.11 | 1.08 |
| 5 | 1919 | 129 | 1.08 | 1.06 |
| 6 | 1541 | 89 | .93 | .83 |
| 7 | 407 | 20 | .53 | .06 |
| Total Recovery | 1326 mg | | | |
| % | 95.7 | | | |

Table III

Distribution of $PGB_X$ in Sephadex LH-20

Fractions: 3rd MEC

| Fraction | $\bar{M}n$ | Wt (mg) | $K_a$ | $K_i$ |
|---|---|---|---|---|
| 1 | 2862 | 98.4 | .87 | .89 |
| 2 | 2364 | 209.1 | 1.18 | 1.18 |
| 3 | 2209 | 200.3 | 1.09 | 1.26 |
| 4 | 2008 | 115.5 | 1.06 | 1.23 |
| 5 | 1886 | 43.3 | 1.02 | 1.26 |
| 6 | 1466 | 22.9 | .93 | .91 |
| 7 | | 2.43 | .17 | |
| Total Recovery | 691.9 mg | | | |
| % | 101.9 | | | |

Table IV

The Weight Percent Distribution of $PGB_x$ in Fractions Separated by Repetitive Sephadex LH-20 MEC

| | MEC Run | | |
|---|---|---|---|
| Fraction | #1 | #2 | #3 |
| 1 | 13.1 | 10.7 | 14.2 |
| 2 | 19.9 | 23.8 | 30.2 |
| 3 | 41.1 | 27.5 | 29.0 |
| 4 | 12.1 | 20.1 | 16.7 |
| 5 | 18.9 | 9.7 | 6.3 |
| 6 | 16.4 | 6.7 | 3.3 |
| 7 | 6.6 | 1.5 | .4 |

Table V

$A_{\lambda 243nm}/A_{\lambda 310nm}$
of Fractions Separated by Repetitive MEC

| Fraction | 1st MEC | 2nd MEC | 3rd MEC |
|---|---|---|---|
| 1 | 8.14 | 10.42 | 10.22 |
| 2 | 6.85 | 9.50 | 9.00 |
| 3 | 5.58 | 8.08 | 9.89 |
| 4 | 4.96 | 7.67 | 8.60 |
| 5 | 4.12 | 6.20 | 7.33 |
| 6 | 3.55 | 5.05 | 6.23 |
| 7 | --- | 4.33 | 4.78 |

# B I B L I O G R A P H Y


1. Polis, B. D., Grandizio, A. M. and Polis, E.: Some In vitro and In vivo Effects of a New Prostaglandin Derivative. Neurohumoral and Metabolic Aspects of Injury. Adv. in Exper. Med. and Biol., Plenum Press, New York 33:213-220 (1973).

2. Polis, B. D., Kwong, S., Polis, E., Nelson, G., and Shmukler, H. W.: Studies on $PGB_X$, A Polymeric Derivative of Prostaglandin $B_1$: I-Synthesis and Purification of $PGB_X$. Physiol. Chem. and Physics 11:109 (1979).

3. Ohnishi, S. T. and Devlin, T.: Calcium Ionophore Activity of a Prostaglandin $B_1$ Derivative ($PGB_X$). Biochemical and Biophysical Research Communications 89:240 (1979).

4. Angelakos, E. T., Riley, R. L., and Polis, B. D.: Recovery of Monkeys from Cardiogenic Shock After Myocardial Infarction with Ventricular Fibrillation. Effects of $PGB_X$, Report No. NADC-77308-60 (1977).

5. Kolata, R. J.: The Effect of $PGB_X$ on Neurological Recovery from Cerebral Ischemia in Rabbits. Masters Thesis, Univ. of Penna. Veterinary School (1977).

6. Moss, G.: Immediate Restoration of CNS Autonomic Cardiopulmonary Control. Survival of "Lethal" Cerebral Hypoxia by Treatment with $PGB_X$. Surgical Forum 29:535B (1978).

7. Polis, B. D., Polis, E. and Kwong, S.: Protection and Reactivation of Oxidative Phosphorylation in Mitochondria by a Stable Free Radical Prostaglandin Polymer ($PGB_X$). Proc. Nat'l Acad. Sci. 76:1598 (1979).

8. Shmukler, H. W., Soffer, E., Kwong, S. F., Zawryt, M. G., Feely, W., and Polis, E.: Studies on $PGB_X$: Isolation of a $PGB_X$ with Reduced Inhibitor Content, Report No. NADC-79183-60 (1979).